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HEPATOPROTECTIVE AND CYTOTOXIC POTENTIAL OF ETHANOLIC LEAF EXTRACT OF *Polygonum chinense* L.

MANOJ KUMAR DAS

Department of Biotechnology, Cotton College, Guwahati, Assam, India

ABSTRACT

Medicinal plants and their products play a vital role for the development of new drugs. The study is an initiative to establish the medicinal value of *Polygonum chinense* L., a member of Polygonaceae family. The possible hepatoprotective effects of ethanolic leaf extract of *Polygonum chinense* (ELEPC) on paracetamol (acetaminophen) induced acute liver injury was studied in Swiss albino mice. The experimental doses of 250 mg/kg body weight per oral and 500 mg/kg body weight per oral were selected based on acute oral toxicity test. The ELEPC could significantly lower the paracetamol induced elevated level of mice serum alanine aminotransferase (ALT), aspartate aminotransferase (AST) and alkaline phosphatise (ALP) and total bilirubin levels in dose dependent manner. Statistical significance of data was evaluated by one way Analysis of Variance (ANOVA) followed by Dunnett test at $p<0.05$ and $p<0.01$. The histopathologic profile of treated animals depicted hepatoprotective effect of ELEPC. The cytotoxic potential of ELEPC was assessed based on the inhibition of hatching of the cyst (hatchability assay) and brine shrimp lethality assay. The ELEPC was found to be effective against brine shrimp with LC_{50} of 180µg/ml and 140µg/ml in hatchability and lethality assay respectively. ELEPC provide protection against paracetamol induced liver injury and had established its cytotoxic potential which may be targeted for development of new therapeutics.

KEYWORDS: *Polygonum chinense*, Paracetamol, Acetaminophen, Hepatoprotective, Cytotoxic, Brine Shrimp

INTRODUCTION

Assam, the 'Gateway to North-East India' is very rich in natural flora and fauna due to suitable agro-climatic conditions. A huge variety of medicinal plants and herbs of good commercial value can be spotted here but a bare minimum of these are currently investigated. The present study aims at relatively less explored plant *Polygonum chinense* L., commonly called Mountain knotweed, Chinese knotweed, hill buckwheat or Madhusoleng or Soleng in Assamese, known to possess some important bioactivities. *P. chinense* can be found in Asian countries like Indonesia, Malaysia, India, Myanmar, Nepal, Thailand and Vietnam. It is a fast growing dicotyledonous, rhizomatous, much branched, ascending perennial herb. *P. chinense* is used by various ethnic communities for different medicinal properties like gastro protective (Ismail *et. al*, 2012), antibacterial and antifungal (Maharajan et al., 2012), counteract dyspepsia (Sajem and Gosai, 2006). *Polygonum chinense* reported to possess coumarin compound, 8-methyl octahydrocoumarin (antimicrobial, antioxidant, anti-inflammatory) and 1-2-Benzenedicarboxylic acid, mono [2-ethyl hexyl] ester (antimicrobial) (Bagavathi and Ramasamy, 2012). The presence of these compounds in *Polygonum chinense* justifies the use of the plant for various ailments by traditional practitioners. It may have anticancer, chemo preventive, pesticide, anti-tumor and sunscreen properties as it contains squalene as its major phytocomponent (Bagavathi and Ramasamy, 2012).

Paracetamol or acetaminophen is a potent hepatotoxic agent and is used to investigate hepatoprotective activity on various experimental animals (Senthilkumar et al., 2014; Ozougwu and Eyo, 2014). Hepatic damage results in increased serum glutamic oxaloacetic transaminase (SGOT) or aspartate aminotransferase (AST) and serum glutamic pyruvic transaminase (SGPT) or alanine aminotransferase (ALT) and alkaline phosphatase (ALP). The elevation of concentration of these enzymes is generally regarded as one of the sensitive markers of hepatic damage (Maheswari et al., 2014). Although serum levels of both AST and ALT become elevated whenever there is occurrence of diseases affecting liver cell integrity, ALT is the more liver-specific enzyme. Serum elevation of ALT activity is rarely observed in conditions other than parenchymal liver diseases like cirrhosis, carcinoma, hepatitis, obstructive jaundice etc. Moreover, elevation of ALT level persists longer than do those of AST activity. Elevation of alkaline phosphatase (ALP) in serum or plasma is mainly associated with hepatitis, billiary obstructions however also occur in hyperparathyroidism, steatorrhea and bone diseases.

The brine shrimp lethality assay was introduced by Michael et al., 1956. It is based on the ability to kill laboratory-cultured *Artemia* nauplii brine shrimp. The assay is proved to be a useful tool for preliminary assessment of toxicity, and it has been applied to test toxicity of plant extract (Solís et al., 1993; McLauglin et al., 1991). Another assays based on the inhibition of hatching of the cyst have also been applied (Migliore et. a1997).

MATERIALS AND METHODS

Test Material

Leaves of the plant *Polygonum chinense* Linn. were collected from Maligaon area of Guwahati, Assam, India during January-February, 2013. Taxonomic authenticity was confirmed by referring to herbarium specimen at Department of Botany, Cotton College, Guwahati and a voucher specimen was deposited. Freshly collected leaves were washed with tape water, rinsed in distilled water and surface-sterilised. The leaves were shade-dried for 4 weeks and grinded to a coarse powder using a mechanical grinder. 50gm of the powdered leaves was refluxed with 95% ethanol. The dark green coloured extract obtained was made free of solvent using rotary vacuum evaporator and stored at 4˚C until analysed.

Preliminary Phytochemical Screening

The ELEPC thus obtained was subjected to phytochemical screening to test the presence of alkaloids, flavonoids, free amino acid, phenolic compounds, reducing sugar, saponins, tannins and terpenoids by the methods of Trease and Evans (1983).

Hepatoprotective Assay

Randomly bred Swiss albino mice *(Mus musclus)* were obtained from Department of Zoology, Gauhati University, Guwahati, Assam, India. The mice were maintained in the animal house, Department of Biotechnology, Cotton College, Guwahati and sacrificed as per the guidelines of Animal Ethical Committee of G. U. (Regd. No.902/AC/05/CPCSEA). Both male and female mice of 5-6 weeks of age weighing about 20$\pm$ 2g were used. The animals were acclimatised for 1 week under laboratory conditions. Acute oral toxicity test in mice was carried out for selection of appropriate dose as per the method of Lorke, 1983 (results not shown here). Based on these, two working concentrations of 250mg/kg and 500 mg/kg bodyweight of leaf extract were selected.

The animals were randomly assorted into the following five groups of six mice each and treated for 7 days:

Group I: Animals received normal pellet diet and water *ad libitum.*

Group II: Animals received a normal pellet diet with single dose of 1000mg/kg bodyweight of paracetamol (acetaminophen) and served as negative control.

Group III: Animals received a normal pellet diet with single dose of 1000mg/kg bodyweight of paracetamol and 250mg/kg bodyweight of extract for 7days.

Group IV: Animals received a normal pellet diet with single dose of 1000mg/kg bodyweight of paracetamol and 500mg/kg bodyweight of extract for 7days.

Group V: Animals received a normal pellet diet with single dose of 1000mg/kg bodyweight of paracetamol and 25mg/kg bodyweight of Silymarin for 7days and served as positive control.

At the end of the experiment, blood samples were collected from the tail tips of overnight fasted mice in sterilized dry centrifuge tubes. The clear serum was separated by centrifugation at 2500 rpm for 10 minutes and subjected to biochemical investigations for various liver function parameters. ALT and AST were assayed using standard kits manufactured by Crest Biosystems, Goa, India. ALP kit was manufactured by Siemens Ltd., Gujarat, India. The levels of total protein and total bilirubin in the serum were estimated using standard commercial kits from SPAN India Ltd, Surat, India. Paracetamol was from CIPLA Ltd., Himachal Pradesh, India and Silymarin was from Panacea Biotech Ltd., New Delhi, India.

All the results are expressed as mean $\pm$ S. D. of 6 animals. Statistical differences between the experimental groups were determined by one way Analysis of Variance (ANOVA) followed by Dunnett test at 0.05 and 0.01 significance level using GraphPad Prism ver. 5.03, San Diego, California, USA.

Histopathological Studies

The liver was excised, washed using normal saline and fixed in 10% buffered neutral formalin for 48 hours. Further, the liver from each animal was dehydrated in alcohol and embedded in paraffin. Microtome sections (5µm thickness) were prepared from each liver sample, stained with haematoxylin- eosin (H&E) and examined microscopically for the evaluation of histopathologic changes.

Determination of Cytotoxic Activity

To study the cytotoxicity of *P. chinense* leaf extract, brine shrimp hatchability assay and brine shrimp lethality assay were conducted. *Artemia salina* (brine shrimp) cysts were purchased via www.MakeMyHobby.com (invoice no. 12317). Various concentration of ELEPC from 20µg/ml, 40µg/ml, 60 µg/ml,.....to 300µg/ml were tested. Similar experimental doses have been used in earlier studies (Parvin et al., 2012).

Brine Shrimp Hatchability Assay

Brine shrimp cysts were added to a glass beaker containing sterile simulated sea water. The cysts were hatched under continuous illumination and strong aeration. After 2 hours, two aliquots of 250µl were placed in two separate glass vials; one containing ELEPC along with sea water and other vial contains only sea water (control). Both the vials were incubated under similar conditions and illuminated with gentle shaking. After 12, 24 and 48 hour of exposure, the free nauplii were calculated (Hamid et. al, 2011). The experiment was performed in triplicates. The percentages of hatchability were calculated by comparing the number of free nauplii in each treatment with the number of free nauplii in the control. Later the percentage of hatch inhibition (%HI) was calculated as:

% HI = % hatchability in the control - % hatchability in each treatment.

Brine Shrimp Lethality Assay

Approximately 12 hour after hatching of the brine shrimps, the free-swimming, pink coloured, phototropic nauplii were collected with a micropipette and concentrated in a glass vial. 10 nauplii were transferred to each test tube containing various concentrations of ELEPC and to a control test tube without plant extract. Volume of each test tube is made up to 10 ml using simulated seawater and incubated under similar conditions of temperature and illumination. The mortality was determined after 12hr and 24 hr of exposure under constant illumination. The numbers of survived larvae were counted and percentage of deaths was calculated. The experiment was performed in triplicates. Larvae were considered dead if they did not exhibit any controlled forward movement during 30 seconds of careful observation. The mortality observed may be attributed to bioactive compounds because in any case, hatched brine shrimp nauplii can survive up to 48 h without food (Lewis, 1995). However, in cases where control deaths were detected, the percentage of mortality (% M) was calculated as:

% Mortality = % survival in control - % survival in treatment

Based on the percentage mortality, the LC_{50} of ELEPC was determined using Probit values of Finney. If death occurred in the control tubes at the end of the treatment, the percentage of death were corrected using Abbott's formula.

RESULT AND DISCUSSIONS

Preliminary Phytochemical Screening

Preliminary phytochemical investigation was considered before examining hepatoprotective and cytotoxic potential of ethanolic leaf extract of *P. chinense*. ELEPC showed the presence of many groups of compounds (Table 1); these compounds are very important and may be responsible for various bioactivity of the plant including hepatoprotective and cytotoxic behaviors. Flavonoids and saponins are well known for its antioxidant and hepatoprotective activities (Junei et al., 2006).

Table 1: Phytochemical Content of ELEPC

Test	Result
Alkaloid	Absent
Free amino acids	Absent
Flavonoids	Present
Phenolic compounds	Present
Reducing sugars	Present
Saponins	Absent
Tannins	Present
Terpenoids	Present

Hepatoprotective Assay

The selected high (500mg/kg b.w.) and low dose (250mg/kg b.w.) of ELEPC were tested for their effects on liver function parameters in Swiss albino mice. Serum levels of aspartate aminotransferase (AST), alanine aminotransferase (ALT) and alkaline phosphatise (ALP) of all the treated animal groups are tabulated below (Table 2). Rise in serum levels of AST, ALT and ALP were noted when the animals treated with 1000mg/kg bodyweight of paracetamol. Paracetamol is a known antipyretic and analgesic which produces hepatic necrosis in high doses (Firdous et al., 2012). Subsequent feeding of extract has been able to bring down the elevated serum levels of AST, ALT and ALP in dose dependent manner. ELEPC regulated the total bilirubin and total protein levels of the test animals positively during the experimental period as well

(Table 2). All the liver function parameters tested were recovered to normal levels by the higher dose of ELEPC and remained comparable to the positive control group treated with established hepatoprotective drug silymarin.

Table 2: Hepatoprotective Effect of ELEPC against Paracetamosl Induced Toxicity

Group	Treatment Duration	AST (IU/L)	ALT (IU/L)	ALP (IU/L)	Total Bilirubin (mg/dL)	Total Protein (g/dL)
I (normal mice)	7 days	76.66±4.25	43±5.45	146.21 ± 1.28	1.50± 0.02	7.83±0.36
II (Negative control)	7 days	160±4.28^{b}	115.12 ± 3.91^{b}	252.31 ±2.30^{b}	2.72± 0.01^{a}	6.72±0.12^{a}
III (250mg/kg b.w. of ELEPC)	7 days	112.31 ±4.22^{b}	67.37 ± 5.34^{b}	180.42 ± 5.33^{b}	1.88± 0.02^{a}	7.21±0.17^{a}
IV (500mg/kg b.w. of ELEPC)	7 days	84.33±5.^{b}	51.35 ± 5.25^{b}	158.44 ± 1.84^{b}	1.72± 0.01^{a}	7.05±0.08^{a}
V (Positive Control-25mg/kg b.w. of Silymarin)	7 days	81.44±3.75^{b}	45.6 ± 3.87^{b}	151.12 ± 2.65^{b}	1.60± 0.02^{a}	7.75±0.12

Values expressed as mean ± S. D. of 6 animals. 'a' and 'b' represent significant changes at $p < 0.01$ and $p < 0.05$ respectively when compared to control (one way ANOVA followed by Dunnett test).

Histopathological Studies

Liver sections of mice treated with paracetamol depicted intense centrilobular necrosis, vacuolization and macro vesicular fatty changes (Figure 1b). Normal tissue architecture was evident in the liver sections of silymarin treated animals (Figure 1c). Animals treated with 250mg/kg body weight of ELEPC showed normal hepatic cords with moderate fatty infiltration of liver (Figure 1d). Liver section of mice treated with higher dose (500mg/kg b.w.) of extract demonstrated significant protection against paracetamol induced liver damage. It was apparent by the presence of normal hepatic cords, absence of necrosis, fatty infiltration and reformation of tissue architecture (Figure 1e).

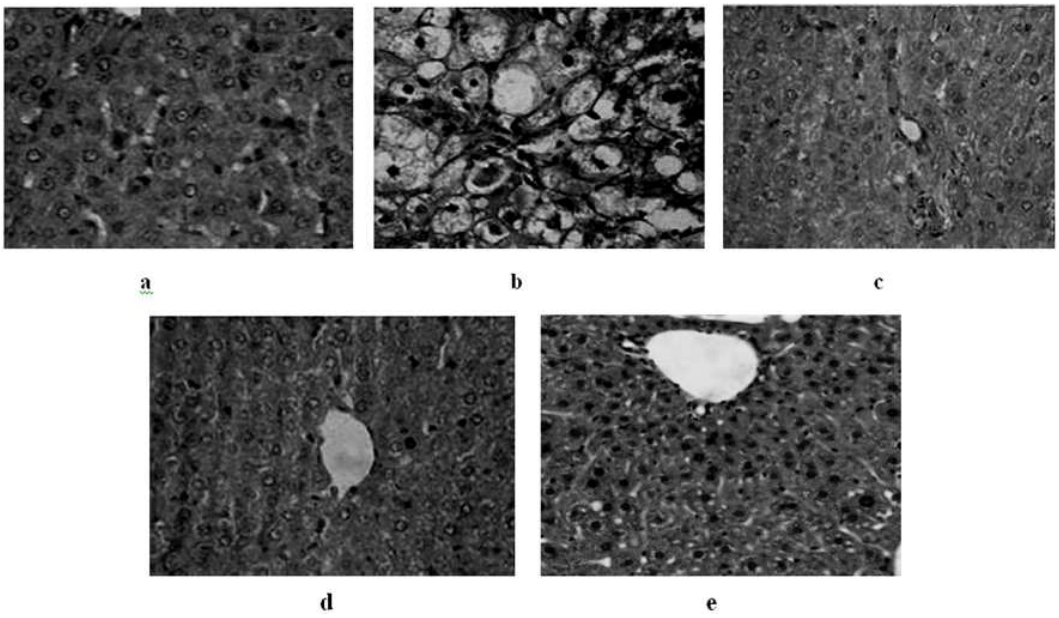

Figure1: Histopathologic Profiles-Normal Mice (a); Liver Sections of Animals Treated with Paracetamol (b); Liver Sections of Silymarin Treated Animals (c); Animals Treated with 250mg/kg b. w. of ELEPC (d); Animals Treated with 500mg/kg b.w. of ELEPC. Original Magnification: 100X

Brine Shrimp Cytotoxic Assay

The brine shrimp bioassays represent a simple, rapid and low cost tool for testing cytotoxicity of plant extract (Persoone and Wells, 1987). Hatchability assay was carried out after 12, 24 and 48 hours of inoculation of the young nauplii in the glass vials. ELEPC was active against the brine shrimp at a minimum concentration of 40µg per ml. after 12 hours of incubation and increased in dose dependent and time dependent manner. A strong hatchability inhibition was observed at 200µg per ml (52.11%) with maximum hatchability inhibition at 300 µg per ml (74%) after 24 hours of exposure. ELEPC has shown strong hatchability inhibition at 140µg per ml. (53.25%) after 48 hours with a maximum of 93% at 300µg per ml. The LC_{50} of ELEPC was found to be 180µg per ml. after 24hours and 48 hours of incubation based on Probit values of Finney. Brine shrimp was found to be more vulnerable to ELEPC at the early developmental stages as observed in case of other toxic compounds (Sleet and Brendel, 1985).

Table 3: Cytotoxic Potential of ELEPC Observed in Brine Shrimp Assays

Drug conc. (µg/ml.)	Percent Hatchability Inhibition at 12 hours	Percent Hatchability Inhibition at 24 hours	Percent Hatchability Inhibition at 48 hours	Percent Mortality at 12 hours	Percent Mortality at 24 hours
20	0	5.00	13.55	0	14.00
40	7.2	9.15	24.22	11.22	17.25
60	12.22	14.33	30.15	20.5	29.12
80	15.5	19.55	33.45	26.1	31.4
100	19.65	23.2	41.5	28.67	35.00
120	35.15	38.4	46.33	32.15	40.33
140	37.22	42.00	53.25	49.00	60.25
160	41.5	44.33	57.27	54.45	63.45
180	40.7	49.65	60.45	57.25	66.67
200	49.1	52.11	65.55	61.11	69.12
220	55.55	56.00	72.11	68.3	80.42
240	59.33	61.25	78.4	71.23	88.11
260	64.2	68.44	84.12	76.00	94.53
280	67.45	71.5	89.00	76.00	100
300	68.1	74.00	93.00	81.25	100

A high lethality was observed at 160 µg per ml (54.45%) after 12 hours of ELEPC treatment whereas 140µg per ml. was sufficient to show a high lethality of 60.25% after 24 hours. ELEPC had shown increase in lethality significantly up to 260µg per ml. and attained 100% lethality at 280µg per ml. after 24 hours of incubation. At this stage in their life cycle, the nauplii had reached their second and third instar and exhibit their greatest sensitivity to test compounds (Lewis, 1995). The LC_{50} of ELEPC was 140µg per ml. based on Probit values of Finney. The hatchability test depicted cytotoxicity of ELEPC similar to the lethality assay, but appeared less sensitive. A good correlation can be observed between brine shrimp bioassays and detection of antitumorigenic compounds in plant extracts (Mackeen et al., 2000; Solís et al., 1993).

CONCLUSIONS

Medicinal plants are an integral component of research in development of pharmaceuticals. Hepatoprotective role of ethanolic leaf extract of *Polygonum chinense* L. was recorded for the first time against acetaminophen induced toxicity in Swiss albino mice. The study also suggested the safety aspects of the modulator at selected dose levels. Cytotoxic potential of the extract was ascertained when evaluated by brine shrimp bioassays and can be extended to investigate its antitumor activity in future.

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